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PROVITAMIN D POTENCIES,
ABSORPTION SPECTRA, AND
CHEMICAL PROPERTIES OF
HEAT-TREATED CHOLESTEROL

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE BIO-
LOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY AND PHARMACOLOGY

1932

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MILICENT LOUISE HATHAWAY

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PLATES 1 AND 2

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The biochemical and spectroscopic studies by Bills, Honeywell, and MacNair (1) on purified cholesterol first showed that commercial cholesterol purified by various methods yielded a cholesterol, apparently free from ergosterol, which still possessed provitamin D activity. The work of Koch, Koch, and Ragins (2) in these laboratories confirmed these results and also showed that on heating this purified material with little or no oxygen present at temperatures slightly above the melting point of cholesterol for periods of from 1 to 3 hours, the activatability was materially increased.

Since 1929 further work has been done along somewhat similar lines. Montignie (3) considers activation due to the formation of a small amount of an isomer of cholesterol rather than to an impurity such as ergosterol. Kaishio and Gardner (4), although they discount Montignie's conclusions, do find changes in absorption spectra and color tests as a result of heat treatment on cholesterol. Schönheimer (5) has also shown that heat changes cholesterol, his theory being the simultaneous formation of sterols of the ergosterol and dihydrocholesterol types. No biological studies on their products are reported by any of these workers. Mouriquand and Leulier (6) show that irradiation of the sterol from *Helix pomatia*, a type of snail, yields a product with antirachitic properties; and Labbé, de Balsac, and Lerat (7) show similar properties for one of the theosterols isolated from cacao bean shells. However, none of these investigators appears to have

treated his sterol to insure destruction or removal of traces of ergosterol which may be present.

In this investigation we are reporting further studies involving the effects of heat on cholesterol, resulting in its increased provitamin D activity.

Preparation of Sterol Products—Commercial cholesterol from The Wilson Laboratories was purified by the bromine treatment described by Bills, Honeywell, and MacNair (1). The original material showed ergosterol bands, and after irradiation by ultraviolet light was antirachitic in doses of 0.1 mg. per rat per day. After the purification treatment it showed no absorption bands, little or no general absorption, and a limiting antirachitic dose of 10 mg. of the irradiated product per rat per day. The non-irradiated purified product was the starting material for all further studies reported here.

The purified cholesterol was placed in a 2×11 cm. test-tube fitted with a ground glass stopper which carried two outlet tubes. The ground glass joint was lubricated and sealed with cholesterol in ether solution. In some samples, the air in the tube was completely displaced by pure dry nitrogen and the outlet tubes were closed before it was heated; in other cases the oxygen of the air was not removed from the tube and the outlet tubes were left open during the heating. In all cases the tube was introduced into a bath of Wood's metal previously heated to the desired temperature. Two temperatures, 200° and 300° , were selected, as it had been found in these laboratories (2) that a temperature around 200° caused increased activatability and 300° was used by Diels and Linn (8) in the preparation of their β -cholesterol which we thought might be an active isomer. At the close of a definite heating period at constant temperature, the tube was removed from the bath, opened, and cooled until the sterol had solidified. The sterol was removed by treating it with small portions of alcohol in a boiling water bath and filtering the hot solution from undissolved material. Much of the sterol crystallized when the alcohol solution was cooled to 0° . The crystals were separated by suction filtration, rapidly washed with cold 95 per cent alcohol, and dried in air. These crystals constituted Fraction A. The filtrate and washings evaporated nearly to dryness on the steam bath gave a product, Fraction B, which had a tendency to be

more resinous and yellow in color. In an attempt to concentrate further the provitamin D activity, Fractions A and B were again recrystallized from hot alcohol. These fractions are designated in Table I as Fractions A-1, A-2, B-1, and B-2.

The material insoluble in hot alcohol was called Fraction C. It was easily soluble in benzene and chloroform, but difficultly soluble in ether. On recrystallization from benzene by precipitation with alcohol, it gave a very uniform product consisting of fine white crystals melting at 194.5° . These properties are all characteristic of the dicholesteryl ether described by Mauthner and Suida (9).

Biological Assay—Young rats weaned when 21 days old (weighing at least 35 gm.) were kept in a dark room for 14 days on the McCollum Diet 3143 (10). The material to be tested for curative properties was added to this diet for a subsequent 10 day period.

The activation was carried out by exposing a thin layer of a known amount of the dry crystals in flat Petri dishes under a Cooper Hewitt quartz mercury arc lamp at a distance of 40 cm. for a period of 35 minutes. The irradiated material was then incorporated with a known amount of the rachitic diet and fed in definite amounts to rats in small individual cages according to the method of Koch, Koch, and Ragins (2). Regularly three animals were used for each level, from three to six more being used in those cases in which particularly irregular results were obtained.

At the end of the 10 day test period the rats were killed and the proximal ends of the tibiae were examined for the degree of calcification by the Shipley line test.

Provitamin D Potencies of Fractions from Heated Cholesterol—Preliminary spectrographic studies showed that preparations possessing greater general absorption in the ultra-violet range are obtained when the purified cholesterol is heated at 300° than at 200° . As we hoped to find increased provitamin D activity to parallel this greater absorptive property, we first studied Fractions A, B, and C obtained by heating cholesterol as indicated above at 300° for 20, 60, and 120 minutes. Preliminary biological assays on the irradiated products indicated higher provitamin D activity on heating for 120 minutes than for shorter periods; therefore fractions thus obtained were studied in more detail and the

effect of longer heating, for 240 minutes, was also determined. Daily doses of 0.1, 0.5, and 1.0 mg. of the irradiated product were fed to rats, higher doses being used later in cases in which rickets persisted on the 1.0 mg. dose. The fractions of sterol heated at 300° did not show as great provitamin D activity as had been found in these laboratories in the earlier studies (2), so that samples were prepared by heating the purified cholesterol at 200° for 120

TABLE I
Summary of Antirachitic Potencies of Irradiated Fractions Obtained from Heated Cholesterol

Heated before irradiation at	Time	Heated (+ or - O ₂)	Fraction*	Order of 2+ curative dose
°C.	min.			mg.
300	120	—	A-1	0.5
300	120	—	A-2	1.0
300	120	—	B-1	1.0
300	120	—	B-2	>1.0
300	120	+	A	<0.5
300	120	+	B	<1.0
300	240	—	A	<5.0 >1.0
300	240	—	B-1	>1.0
300	240	—	B-2	>4.0
300	120 or 240	—	C	>50.0
200	120	—	A	0.5
200	120	—	B	1.0
200	120	+	A	0.1
200	120	+	B	0.5

* Fraction A, crystallized from alcohol; Fraction A-1, Fraction A recrystallized from alcohol; Fraction A-2, from filtrate from recrystallization of Fraction A; Fraction B, from first alcohol filtrate; Fraction B-1, Fraction B recrystallized from alcohol; Fraction B-2, from filtrate from recrystallization of Fraction B; Fraction C, dicholesteryl ether, insoluble in alcohol.

minutes both in an atmosphere of nitrogen and in the presence of oxygen. It is interesting to note that at this temperature only alcohol-soluble fractions A and B were found; no dicholesteryl ether was formed.

A summary of the results of the biological assays at both temperatures is given in Table I. If more tests had been run with a more gradual gradation of doses, more accurate determinations of the potency of the fractions could have been made, but the

following observations can be made from the results as indicated; all heated samples (except Fraction C) showed higher provitamin D activity than the purified cholesterol (which required 10 mg. per day) and 200° is more effective in producing the increase than 300°; the fractions crystallizing from alcohol showed higher potency than the more soluble fractions prepared from the filtrates; the presence of oxygen in the tube during the heating still further increased the provitamin activity; and Fractions C obtained at 300° both after 120 and 240 minutes of heating were consistently inactive, even in doses as high as 50 mg. per day.

Spectroscopic Studies—The method used was essentially that employed by Koch, Koch, and Lemon (11), with the following substitutions. As a continuous hydrogen spectrum source a 60 cm. water-cooled tube of inverted TT type was used, in which large internal aluminum electrodes were placed close to the walls to permit dissipation of heat. The hydrogen was maintained at several mm. pressure by the presence of a reservoir of liter capacity. The current for excitation was taken from the 20,000 volt secondary of a 60 cycle transformer, the primary of which was connected directly to the 110 volt A.C. mains.¹ The light emitted through the quartz window in the end of the tube was focused by a quartz lens on the slit of a Bausch and Lomb quartz spectrograph No. 2820.

The materials were weighed into 10 cc. volumetric flasks and dissolved in anhydrous ether. The solutions were exposed in quartz Baly tubes for 30 seconds with slit width and length of 0.03 and 5 mm. respectively.

Representative examples of the results of spectroscopic analysis of the various sterol samples are given in Figs. 1 to 4.

Fig. 1 reveals that the Wilson product causes general absorption and also contains traces of ergosterol but that the general absorption factor and the ergosterol are removed in our purified product. On heating the purified cholesterol, a general absorption of greater intensity is produced but no specific absorption bands can be demonstrated unless ergosterol is added. Fig. 2 shows that very pronounced general absorption is produced by Fractions A and B prepared by heating at 300° for 240 minutes, but that practically

¹ This hydrogen tube was furnished through the courtesy of Professor H. B. Lemon of the Department of Physics.

no absorption is possessed by Fraction C, the dicholesteryl ether. Fig. 3 confirms the observations on Fractions A and B, when material is heated for only 120 minutes. Fig. 4, spectra *D* to *I*, shows that Fractions A obtained from heated purified cholesterol, although more potent in provitamin D than the original Wilson product, nevertheless possess less general absorption and no ergosterol bands.

Color Reactions of Our Products—The Rosenheim (12) trichloroacetic acid reaction was applied to our samples.² Fraction A, 120 minutes at 200°, most potent in provitamin D activity, gave no color in 12 hours; other samples heated at 300° changed to crimson in 10 minutes rather than to the blue produced by ergosterol in that interval. These tests confirm the spectroscopic observations, that ergosterol is not formed in measurable amounts when the provitamin D activity of purified cholesterol is increased by heat treatment.

Other Chemical Studies—Chemical tests on the various fractions of heat-treated cholesterol were made in an attempt to determine the extent of modification of the cholesterol and the nature of the change. Only three lines of investigation have been carried out as yet: first, precipitation of the sterol with digitonin and the quantitative determination of the digitonide; second, the removal as the dibromide of unsaturated sterols and subsequent precipitation and quantitative determination of the digitonides of the saturated sterols; third, the estimation of the iodine numbers.

Since cholesterol is quantitatively precipitable as the digitonide, we may assume that the portions of the heat-treated samples *not* precipitable must have been changed but further studies are required to show that all of the precipitable material is unaltered cholesterol. Owing to the small amounts of some of the fractions available, microchemical methods were desirable. Application of the microoxidation method of Bloor (13) and Okey (14) for the determination of cholesterol in blood (as the digitonide) gave us variable results, and, since several other workers (15–18) had also found the method unsatisfactory without modifications, the microgravimetric method of Mancke (19) was used. The results are given in Table II, Column 2. Since the original cholesterol and

² For further details see Loehr, A., Master's thesis, University of Chicago (1931).

our most potent fraction (Fraction A, 120 minutes at 200°), irradiated or non-irradiated, were all recovered 100 per cent as the digitonide and the results did not parallel the increased provitamin D activity, no further studies of digitonin precipitates were made.

If Schönheimer's (5) theory is correct that heat changes the cholesterol simultaneously into sterols of the ergosterol and dihydrocholesterol types, if these two are formed in parallel amounts,

TABLE II
Chemical Studies of Heat-Treated Cholesterol

Sterol	Sterol recovered as			Iodine No.	Order of 2+ curative dose
	Digitonide Mancke method	Dibromide Schönheimer method	Dihydrocholesterol Schönheimer method		
(1)	(2)	(3)	(4)	(5)	(6)
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		<i>mg.</i>
Cholesterol, Wilson Laboratories . . .	99.6	93	0	55.5	0.1
Purified cholesterol		93	0	59.5	10.0
120 min., 300°, Fraction A	69.0	55	1.8		1.0
240 " 300° " "	42.1	<14	2.5		<5 >1.0
240 " 300° " B-1	51.7	39	1.4		>1.0
240 " 300° " B-2	24.9				>4.0
120 " 200° " A	102.6	102	0	58.1	0.5
120 " 200° " irradiated	102.2				0.5
120 min., 200°, Fraction B	87.7				1.0
Pure ergosterol				251	
Purified cholesterol + 2% ergosterol.				61.2*	

* Calculated, 63.3.

and further, if the increased activatability is due to the formation of an ergosterol-like substance, the amount of dihydrocholesterol in the sample should be an index of its provitamin D activity. His method (20) for the determination of small amounts of saturated sterols present in cholesterol was used; *i.e.*, the formation from the cholesterol of its dibromide, its removal by filtration, and the quantitative precipitation from the filtrate by digitonin of the dihydrocholesterol (unaffected by the bromine treatment). As rather large differences were found between the

amounts of dibromide when 50 and 100 mg. samples of the same sterol were used, studies were made on the solubility of the dibromide in the alcohol used as solvent. The average of five 50 mg. samples of dibromide carried through the same conditions of temperature and amounts of solvent used in the experiment showed that 9.6 mg. of the dibromide were dissolved. With this correction, the values given in Table II, Columns 3 and 4, were found.

Since cholesterol from The Wilson Laboratories, purified cholesterol, and our most active fraction (Fraction A, 120 minutes at 200°) showed no evidence of dihydrocholesterol, it was indicated that the dihydrocholesterol content did not parallel the increase in provitamin D activity.

It is striking that in the fraction most active biologically (Fraction A, 120 minutes at 200°) 102 per cent of the sterol was recovered as the dibromide (assuming the sterol was of the same molecular weight as cholesterol), which indicates that the increased biological activity is accompanied by increased unsaturation. Therefore, the iodine numbers of several preparations were determined at 0° by the method of Rolls (21) and the values obtained are given in Table II, Column 5. Although the heat-treated sample was at least 20 times as potent in provitamin D content as the purified product, it had a lower iodine number. The iodine number certainly does not indicate greater unsaturation; however, some other disturbing substance may have been formed which complicates the interpretation of the iodine number estimations.

SUMMARY

Heat treatment of purified cholesterol increases its proantirachitic potency from the requirement of about 10 mg. per day to 0.1 to 0.5 mg. per day. This confirms the earlier work from these laboratories.

The provitamin D activity is produced more effectively at 200° than at 300°. Heating for 240 minutes at 300° materially destroyed the provitamin factor.

The fractions of the heated material which are more difficultly soluble in alcohol showed the most consistent increase in activatability; the more easily soluble fractions showed irregular results but, in general, less potency. Dicholesteryl ether, the alcohol-insoluble fraction, formed only at 300°, is entirely inactive when irradiated and given in doses up to 50 mg. per rat per day.

No spectroscopic evidence of ergosterol bands was found in any of the products of heat treatment. Some showed increased general absorption, but this did not parallel the provitamin D formation and probably is due in part to other products.

Although the Rosenheim trichloroacetic acid color tests were rather unsatisfactory, they did not indicate the presence of ergosterol in our heat-treated samples.

Digitonin precipitation tests showed that from 0 to 75 per cent of the cholesterol is changed by heat treatment into a non-precipitable form, but there is no correlation between this change and the provitamin D activity.

The tests for dihydrocholesterol also showed that much of the cholesterol has been changed; however, no relationship between the amount of cholesterol lost and the provitamin D formation could be established.

The iodine number values obtained show that the purified cholesterol, free from ergosterol, has a higher iodine number than the original Wilson product, which contains ergosterol. A sterol sample (Fraction A, 120 minutes at 200°) obtained in crystalline form from the alcohol solution of purified cholesterol heated for 120 minutes at 200° gave a slightly lower iodine number than the purified cholesterol. Ergosterol itself gives an abnormally high iodine number. When 2 per cent ergosterol is added to pure cholesterol, the iodine number is raised but not to as much as the calculated weighted average of the individual iodine numbers.

Although the fractionation methods used are far from quantitative, we have been able to obtain from heat-treated cholesterol crude fractions of different provitamin D activities, depending on the temperature and time of heating and the solvent used.

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EXPLANATION OF PLATES

PLATE 1

The time of each exposure was 30 seconds.

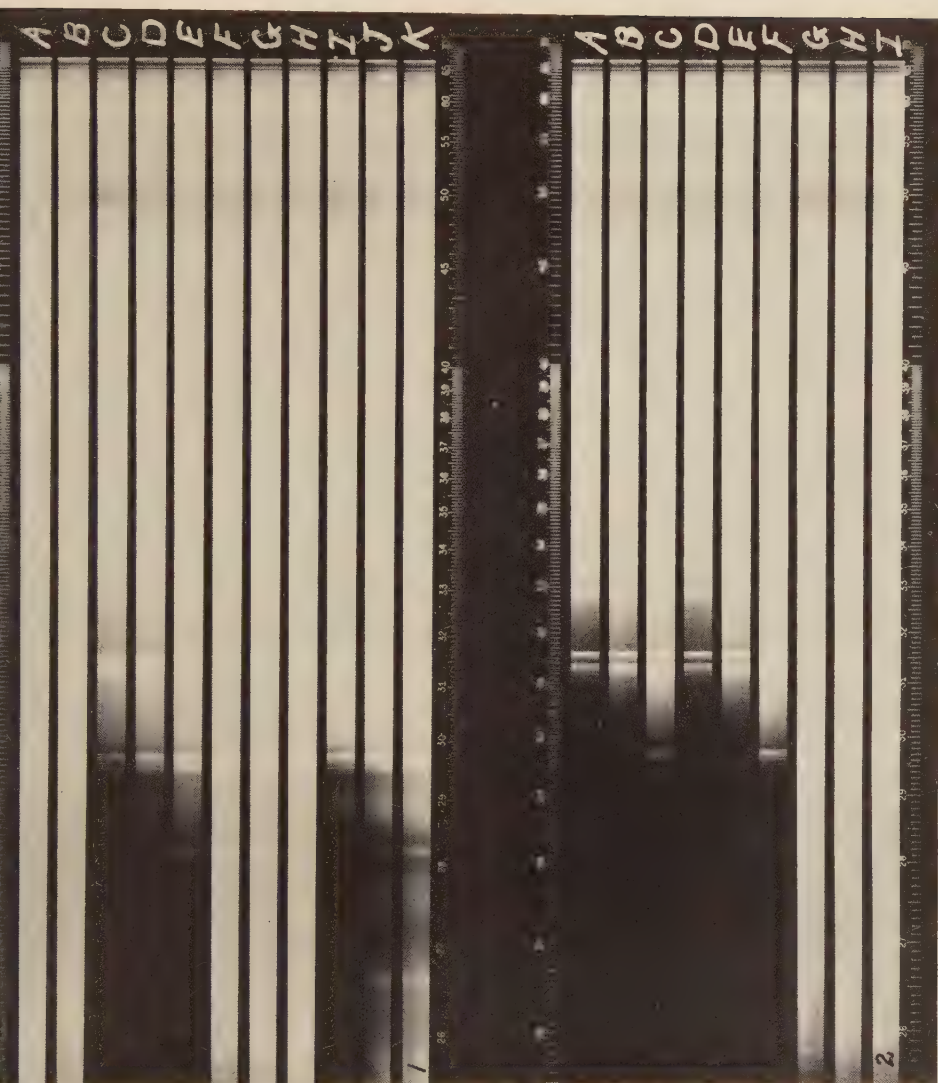
FIG. 1. *A*, empty Baly tube; *B*, anhydrous ether, 20 mm. depth; *C*, 2 per cent Wilson's cholesterol, 20 mm. depth; *D*, same, 15 mm. depth; *E*, same, 10 mm. depth; *F*, 2 per cent cholesterol from the dibromide, 20 mm. depth; *G*, same, 15 mm. depth; *H*, same, 10 mm. depth; *I*, 2 per cent cholesterol from the dibromide plus 0.001 per cent ergosterol, 20 mm. depth; *J*, same, 15 mm. depth; *K*, same, 10 mm. depth.

FIG. 2. *A*, 0.4 per cent Fraction A heated 240 minutes in a nitrogen atmosphere at 300°, 20 mm. depth; *B*, same, 15 mm. depth; *C*, same, 10 mm. depth; *D*, 0.4 per cent Fraction B heated 240 minutes at 300°, 20 mm. depth; *E*, same, 15 mm. depth; *F*, same, 10 mm. depth; *G*, 0.4 per cent dicholesteryl ether, 20 mm. depth; *H*, same, 15 mm. depth; *I*, same, 10 mm. depth.

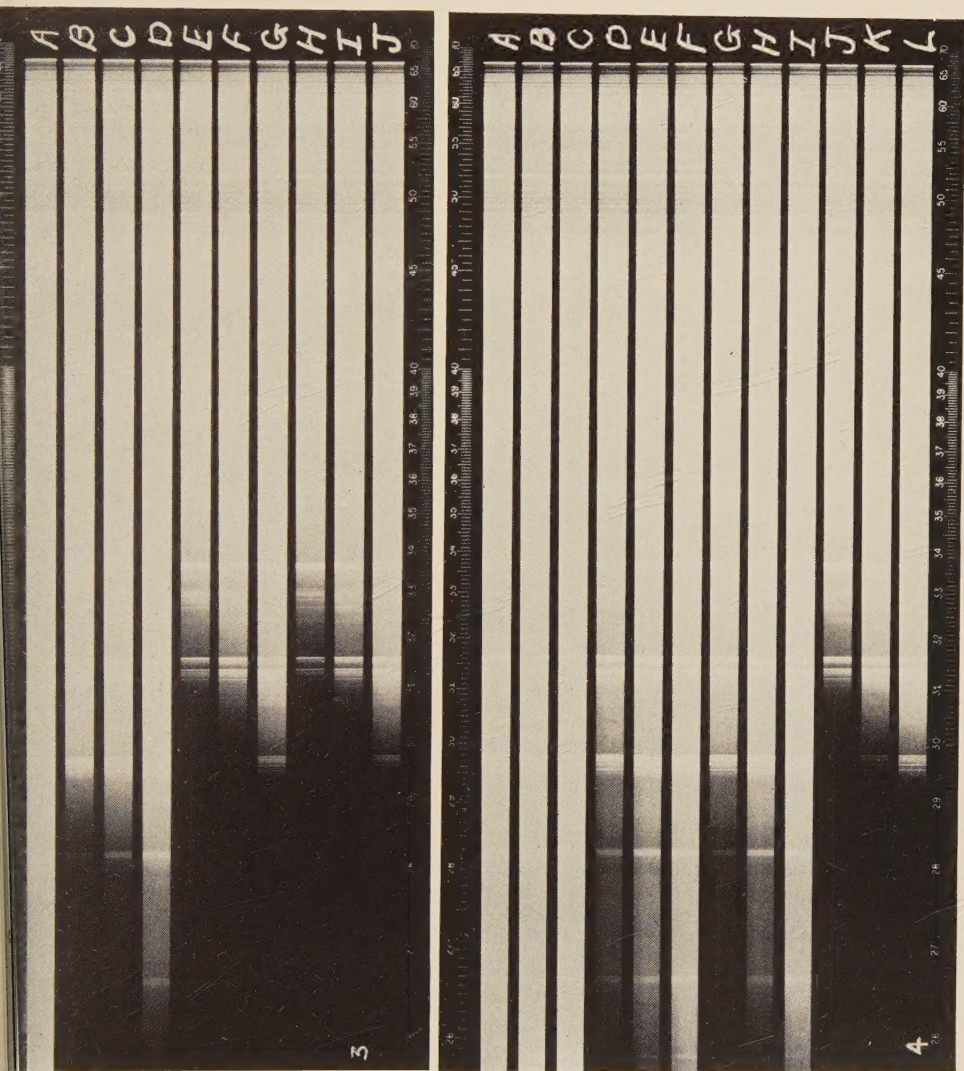
PLATE 2

FIG. 3. *A*, anhydrous ether, 20 mm. depth; *B*, 0.4 per cent Fraction A-1 heated 120 minutes at 300°, 20 mm. depth; *C*, same, 15 mm. depth; *D*, same, 10 mm. depth; *E*, 0.4 per cent Fraction A-2 heated 120 minutes at 300°, 20 mm. depth; *F*, same, 15 mm. depth; *G*, same, 10 mm. depth; *H*, 0.4 per cent Fraction B heated 120 minutes at 300°, 20 mm. depth; *I*, same, 15 mm. depth; *J*, same, 10 mm. depth.

FIG. 4. *A*, 0.4 per cent Fraction A heated 120 minutes in an open tube at 200°, 20 mm. depth; *B*, same, 15 mm. depth; *C*, same, 10 mm. depth; *D*, same material, 2 per cent, 20 mm. depth; *E*, same, 15 mm. depth; *F*, same, 10 mm. depth; *G*, 2 per cent Fraction A heated 120 minutes in a nitrogen atmosphere at 200°, 20 mm. depth; *H*, same, 15 mm. depth; *I*, same, 10 mm. depth; *J*, 0.4 per cent Fraction B heated 120 minutes in a nitrogen atmosphere at 200°, 20 mm. depth; *K*, same, 15 mm. depth; *L*, same, 10 mm. depth.



(Hathaway and Koch: Heat-treated cholesterol)



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